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Secção Autónoma de
Ciências da Saúde

Inês Abrantes
Cravo Roxo

EPIDEMIOLOGIA DE ESTIRPES PRODUTORAS DE β-LACTAMASES

EPIDEMIOLOGY OF β-LACTAMASE PRODUCING ISOLATES

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Epidemiologia de estirpes produtoras de β-lactamases

Epidemiology of β-lactamase producing isolates

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular realizada sob a orientação científica da Doutora Sónia Cristina das Neves Ferreira, Investigadora do Centro de Neurociências e Biologia Celular da Universidade de Coimbra, e co-orientação do Dr. Elmano José da Cruz Ramalheira, Professor Auxiliar Convidado da Secção Autónoma de Ciências da Saúde e Director do Serviço de Patologia Clínica do Centro Hospitalar do Baixo Vouga, E.P.E. – Hospital Infante D. Pedro, Aveiro.

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To my “Grups”, that by knowing how to hold together, teaches me the value of friendship, and fills me with the courage needed in every big challenge.

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Palavras-chave: Bactérias Gram-negativas, Infecções do Trato Urinário, β-lactamase, AmpC

Resumo: As bactérias multirresistentes são um problema emergente por todo o mundo, associado a estadias prolongadas nos hospitais, e ao inerente aumento de custos. As bactérias produtoras de β-lactamases de espectro alargado, amplamente estudadas, e as produtoras de enzimas AmpC, não tão mencionadas, são objecto de grande importância, e a sua epidemiologia deve ser seguida de perto.

A epidemiologia de bactérias produtoras de β-lactamases de espectro alargado de pacientes com mais de 65 anos que recorrem à Urgência com diagnóstico de infecção urinária, bem como a epidemiologia de estirpes produtoras de AmpC, foram avaliadas através do processo de identificação automática Vitek2®. Para os isolados AmpC positivos, testes fenotípicos confirmatórios foram usados para detectar a presença de enzimas AmpC.

Os valores elevados de isolados produtores de β-lactamases de espectro alargado detectados em pacientes com infecção urinária e mais de 65 anos são o maior motivo de preocupação. Uma vez que estes recorrem frequentemente à Urgência ou vivem em lares, estes doentes são potenciais veículos de transmissão destas bactérias multirresistentes. Um problema que estava confinado ao ambiente hospitalar é, hoje em dia, foco de atenção por se encontrar espalhado por toda a comunidade.

Quanto às bactérias produtoras de AmpC, embora sejam menos frequentes do que as produtoras de β-lactamases de espectro alargado, a sua presença pode mascarar a presença do fenótipo característico destas. A avaliação incorrecta induz à prescrição errada de medicamentos e ao consequente surgimento

de estirpes resistentes. Para além disso, existe a possibilidade de se detectarem ambos os mecanismos de resistência na mesma estirpe, aumentando a necessidade de se usarem métodos complementares que as distingam, uma vez que o método automático não é capaz de o fazer. O disco de Cefoxitina é o teste indicado para complementar a identificação da presença da enzima AmpC.

Este estudo mostra a importância de estudar a epidemiologia das β-lactamases, e fornece uma visão realista da sua disseminação pela comunidade, bem como das suas implicações no sistema de saúde da região de Aveiro.

Key words: Gram-negative bacteria, Urinary Tract Infections, β-lactamase, AmpC

Abstract: Multidrug resistant bacteria are an emerging problem worldwide, associated with prolonged stays in the hospital and inherent increased costs. Widely studied ESBL-producers, and the not so considered AmpC-producers are of extreme importance, and its epidemiology should be closely followed.

The epidemiology of ESBL-producing isolates from patients over 65 attending the ER and diagnosed with a UTI, as well as the epidemiology of AmpC-producing isolates were assessed by the Vitek2® procedure of identification. For the AmpC positive isolates, confirmatory phenotypic test were performed, searching for the presence of an AmpC enzyme.

High numbers of ESBL-producing isolates, detected in UTI patients over 65 years old are the main motive of concern, since these are recurrent visitors of hospitals and frequently live in nursing homes, which makes them potential carriers of multiresistant strains. The earlier hospital restricted problem has now become widely spread in the community, and requires further attention.

As for the AmpC, although less frequent than ESBLs, its presence often masks the ESBL phenotype. Misevaluation and false reports induces wrong medication procedures and the consequent emergence of selected resistant strains. Also, the possibility of identifying both resistance mechanisms in one organism has become more common, rising the need of complementary methods to distinguish them, which the automated method is unable to do. Cefoxitin disc was found to be the right complementary test to perform in order to detect these kinds of multiresistant strains.

This study shows the importance of following the epidemiology of β-lactamases, providing a realistic view on its dissemination trough the community and its implications in the health care system in our region.

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List of publications

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Epidemiology of ESBL-producing isolates causing UTI in the elderly (Aveiro, Portugal)

I. Roxo, S. Magalhães, E. Ramalheira, S. Ferreira

Online publication – 25th European Congress of Clinical Microbiology and Infectious Diseases.
(https://www.escmid.org/escmid_library/online_lecture_library/?search=1&current_page=1&search_term=roxo)

(Appendix 1)

Surveillance of MDR Gram-negatives ESBL-producers and carbapenem resistant, in 12 years period (2003-2014) in Aveiro, Portugal

I. Roxo, S. Magalhães, E. Ramalheira, S. Ferreira

Online publication – 25th European Congress of Clinical Microbiology and Infectious Diseases.
(https://www.escmid.org/escmid_library/online_lecture_library/?search=1&current_page=1&search_term=roxo)

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Abbreviations

ATCC - American Type Culture Collection
cAmpC - chromosomal AmpC β-lactamase
CHBV - Centro Hospitalar do Baixo Vouga
DNA - Deoxyribonucleic acid
ER – Emergency room
ESBL – Extended Spectrum β-Lactamases
KPC – *Klebsiella pneumonia* carbapenemase
MIC - Minimal Inhibitory Concentration
MRSA – Methicillin resistant *Staphylococcus aureus*
pAmpC - Plasmid-mediated AmpC β-lactamase
PBP – Penicillin Binding Proteins
RNA - Ribonucleic acid
SMART - Study for Monitoring Antimicrobial Resistance Trends
SSTI – Skin and soft tissue infection
UTI – Urinary tract infection

1. Introduction

1.1 General context

Infections caused by multidrug resistant bacteria are rapidly emerging and are responsible for a worldwide increase of health care-associated costs. Multidrug resistant bacteria can include Gram-negative strains expressing extended spectrum β-lactamases (ESBL), carbapenemases such as *Klebsiella pneumoniae* carbapenemase (KPC) and/or *Enterobacteriaceae* expressing chromosomal AmpC beta-lactamases (cAmpC). (Conen, Frei et al. 2015) The latter isolates are not yet as well studied as other isolates expressing ESBL are, which is disturbing since they are equally a major setback in the treatment of this kind of infections. (Maraskolhe, Deotale et al. 2014)

Several studies report that prolonged stays at the hospital usually leads to infections caused by ESBL producers, and thus reduced rates of clinical response to antimicrobial treatments. (Conen, Frei et al. 2015) Therefore, a more accurate clinical profile of the risk population would be useful in defining strategies to apply to this subset of patients, regarding future savings both in costs and in mortality rates.

One of the most important and most used classes of antibiotics are the β-lactams. Consequently, without surprise, they are plagued with problems of resistance, and the development of new antibiotics (derived by side chain alterations) is not keeping pace with the resistance development. (Worthington and Melander 2013)

For all the exposed above, the epidemiology of ESBL producing isolates in patients attending the emergency room (ER), diagnosed with an urinary tract infection (UTI) and older than 65 years old and the epidemiology of AmpC producing isolates causing infections, in Centro Hospitalar do Baixo Vouga (CHBV) was the focus of this study.

1.2. Bacterial isolates

Each and everyone of us carries 10 times more bacterial cells than human body cells. Despite their vast number, bacteria do not occupy that much space since their size is much smaller than human cells. For a long time, scientists believed that these bacteria, despite their numbers, would do us neither much harm nor much good. (NIH 2012)

However, lately several studies have been shown that bacteria are very important for us, for instance, in maintaining our immune system healthy and by producing chemicals that help us harness energy and nutrients. On the other hand, with the improvement on life conditions, we tend to live longer, which results in an increase of exposition to antibiotherapy through our lives and consequently some of the bacteria we carry can become multiresistant. If most of the time this situation pose no problem, in the right conditions, from the bacteria point of view, it can be fatal to the human being. (Bermon, Petriz et al. 2015)

We tend to forget that bacteria have inhabited the planet for approximately three and a half billion years, longer than mankind, and that they had to adapt on innumerable occasions to toxic substances suddenly introduced into their environments. (Bennett 2008) The systematic development of resistance mechanisms to the antibiotherapy was only one of those adaptations, that have been passed over time as a result of the effect of antibiotherapy selective pressure.

1.2.1. *Enterobacteriaceae*

One of the most common member of the *Enterobacteriaceae* family, is *Escherichia coli*, also the main aerobic commensal bacterial species in the bowel flora yet, in the urinary tract or in blood, it can become pathogenic. In fact, it is the leading Gram-negative pathogen causing nosocomial and community acquired infections. Since *E. coli* easily acquires resistance and is commonly found in many different animal species, its antimicrobial resistance profile is well documented worldwide. (von Baum and Marre 2005; Erb, Sturmer et al. 2007) It is frequently

resistant to aminopenicillins, such as amoxicillin or ampicillin and narrow-spectrum cephalosporins. (Tenover 2006)

Multiresistant isolates of *Enterobacteriaceae* are becoming a common presence within the clinical environments, mainly because bacteria have an impressive capacity to acquire new genes and to rearrange its genetic information (Toleman, Bennett et al. 2006) In general, *Enterobacteriaceae* express low levels of AmpC, but this type of resistance is inducible in response to β-lactams exposure. (Jacoby 2009)

Still, carbapenems (such as imipenem, meropenem and ertapenem) are the most active compounds against ESBL-producing strains of *Enterobacteriaceae* family. Among the most susceptible targets, *E. coli*, *Klebsiella spp*. and *Proteus mirabilis* represent about 98% of these strains, making carbapenems the preferred treatment for infections caused by these bacteria.

However, strains of *Enterobacteriaceae* carrying KPC enzymes, which can render carbapenems uneffective, are emerging in the hospital environment. This situation lead colistin, once been considered toxic, to be taken as a last resort agent for multidrug resistant caused infections. (Perez, Endimiani et al. 2007)

1.2.2 Other than *Enterobacteriaceae* agents

Although Gram-negative bacteria are responsible for almost 70% of all urinary tract infections (Velez Echeverri, Serna-Higuita et al. 2014; Sultan, Rizvi et al. 2015) and *Enterobacteriaceae* are among the most common agents, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus* species are also frequently found as urine pathogens.

Gram-negative organisms are the focus of most of the resistance studies, but equally dangerous are the emerging types of resistance mechanisms found in Gram-positive bacteria. Also, there is another type of pathogen capable of causing

UTIs, *Candida* yeast. Their potential as a urinary tract pathogen is dependent of successful colonization of body sites close to the urinary tract. *C. albicans* is considered normal flora of the body flora and is found in 15%–60% of the population. *Candida* species are unusual causes of urinary tract infections in healthy individuals, but fairly common in inpatients or among patients with predisposing diseases and structural abnormalities of the kidney and collecting system. (Fisher, Kavanagh et al. 2011)

1.3. Infections caused by multidrug resistant bacteria

The innovative introduction of penicillin in the early 1940s was expected to mark the end of infectious diseases, but soon was realized that this was not going to be the case. The emergence of resistant strains was reported just a few years after its use and, since then, resistance to other classes of antibiotics was reported worldwide, mostly due to its overuse.

Although the availability, the controlled and correct use of antimicrobials in developed countries has reduced morbidity and mortality, in developing countries they are empirically applied. The lack of laboratorial results to guide therapy and little information on susceptibility patterns results in many infectious diseases, once easily curable, to become untreatable. (Mshana, Matee et al. 2013)

1.3.1. Urinary Tract Infections

Urinary tract infections are one of the most common causes of hospitalization of patients coming to the emergency room, but also in inpatients. Symptoms of a lower UTI include dysuria without fever, chills or back pain, whereas upper UTI is often suggested by pyelonephritis, loin pain, flank tenderness, fever or other kind of inflammatory response. (Pallett and Hand 2010)

Usually UTI are more frequent in women since the anatomy of the urinary tract in women has some characteristics that may promote it, namely, a longer

urethra and also the proximity to the periurethral region. In elderly, the risk of complicated UTIs is higher in case of prostatic disease in men or post-menopausal women, and thus, those who have higher possibility of developing antimicrobial resistances. Prolonged stays in the hospital, serious complications and extended periods of antibiotic consumption are amongst the main risk factors. (Pallett and Hand 2010)

The choice of agents to treat these infections is now focused on combinations of antibiotics, and broad spectrum agents, such as carbapenems, are being used increasingly as empirical treatment for severe infections, leading to the emergence of organisms producing carbapenemases. Because this kind of resistance is becoming more widespread, it is critical that antimicrobial agents are carefully used as prophylaxis or in asymptomatic bacteriuria. (Pallett and Hand 2010) The antimicrobial oral options for the treatment of complicated UTI caused by ESBL or AmpC producing strains are now limited, especially because of current resistance to trimethoprim and quinolones. The rest of these bacteria remain susceptible to nitrofurantoin (for lower UTIs) and fosfomycin. (Pallett and Hand 2010)

E. coli is one of the most common organisms causing UTIs in the community, and because multidrug resistant strains are more and more frequent, old treatments are struggling to be effective against these emerging UTIs. Other Gram-negative microorganisms, such as urease positive organisms, namely *Proteus spp*, *Morganella morgannii*, *Providencia stuartii and Pseudomonas aeruginosa* are frequently found in indwelling devices, and are also leading causes of UTIs. The resistance developed by these Gram-negative species, especially those that produce AmpC enzymes or extended spectrum β-lactamase (ESBL) poses a major problem in the treatment of these infections. *Enterobacter cloacae*, for example, expresses a chromosomal AmpC β-lactamase that render most of the β-lactams uneffective. Also, Gram-positive cocci, such as methicillin-resistant *Staphylococcus aureus* (MRSA) or *Staphylococcus saprophyticus,* are described

as species causing UTIs. Responsible for about 5% of all UTIs is another frequent colonizing organism *Candida albicans*.

1.3.2. Skin and soft tissue infections (SSTIs)

Being the largest organ of the human body and together with the underlying soft tissue (which includes the fat layers, fascia and muscle), skin represents the majority of all tissue in the body. Skin plays an important role in humans' health: it acts as a tough, flexible and structural barrier to invasion. It is usually colonized with a normal flora, which acts as a competitive inhibitor of pathogenic microorganisms.

Once this barrier is broken or injured, by leg ulcers, burns, surgical or traumatic wounds, it allows colonization by a broad range of bacteria. Although colonization does not require antibiotherapy, infection might, and systemic antibiotics are often used to manage methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA are the major infection agent causing skin and soft tissue infections, as well as β-hemolytic streptococci, usually Lancefield groups A, C and G, with group B occurring in diabetics and in elderly. (Dryden 2010; Mendes and Neves 2012)

Localized pus-producing lesions such as abscesses, carbuncles or wound sepsis are usually staphylococcal, while β-hemolytic streptococci are often responsible for spread infection such as erysipelas or cellulitis. When deeper vascular tissue is compromised, as in diabetic foot or ischemic ulcers, polymicrobial infections may occur, involving both Gram-positive and *Enterobacteriaceae*. *Pseudomonas aeruginosa* is specifically associated with wounds treated with wet bandages. (Dryden 2010)

In chronic patients, with diabetic foot, for example, previously submitted to treatment with antibiotics, infection is likely to be polymicrobial, and require broad-spectrum antibiotic treatment. (Mendes and Neves 2012)

1.4. Antibiotics

The first observation of a bacterial inhibitor was made in early 20th century, when Paul Ehrlich, a German physician, found that an arsenic derived compound, salvarsan, could treat syphilis without harming the patient, as arsenic would do. The Nobel Price in 1908 recognized its importance, and about 30 years later, investigators Domagk and Tréfouël conducted experiments based in this finding that would lead to the discovery of sulphonamides.

In 1928, an accidental finding by Alexander Fleming led to the use of penicillin as the first and major antibiotic in the times to come. While coming back from holidays to his studies on *Staphylococcus aureus*, he realized that the cultures he had forgotten in the bench have been killed by a colony of contaminating *Pennicillium*. The bactericidal effect of penicillin and non toxicity to human made it the most successfully employed antibiotic in the Second World War, and was the first choice against Gram-positive organisms while no resistance was developed.

If the first antimicrobial substances were only produced by fungi and bacteria, nowadays they can be artificially synthesized in pharmaceutical laboratories. An antimicrobial agent is a substance, naturally or artificially obtained, with the capability of killing or inhibiting microorganism growth, active against bacteria, fungi and parasites. The ideal antibiotic should produce this effect with a small amount, with no toxic or collateral effects for the human host.

Antibiotics can be divided according to their biological origin, chemical structure, but also according to the effect on the pathogens. Mainly, they are divided in bacteriostatic – if they can only inhibit microbial growth – or bactericidal – if they kill part of the population, then associated with a minimal inhibitory concentration (MIC).

According to the targeted vital region of the pathogens' cell, antibiotics may also be divided into five categories (Figure 1): cell wall synthesis, protein synthesis, ribonucleic acid (RNA) synthesis deoxyribonucleic acid synthesis (DNA) and intermediary metabolism. (Hooper 2001; Clatworthy, Pierson et al. 2007)

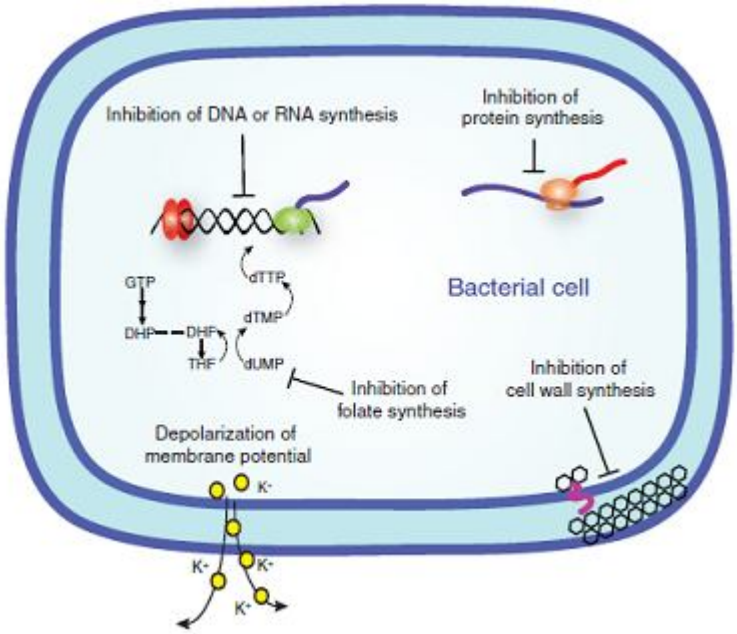

Figure 1 - Traditional targets of antibacterial compounds on the cell vital mechanisms

Exposure to antimicrobial agents has soon produced resistant strains, and efforts to produce new and more efficient ones are an everyday struggle in science. Bacteria can mutate as quickly as new agents are discovered, and that is shown in Figure 2, a timeline of antibiotic deployment and the evolution of antibiotic resistance.

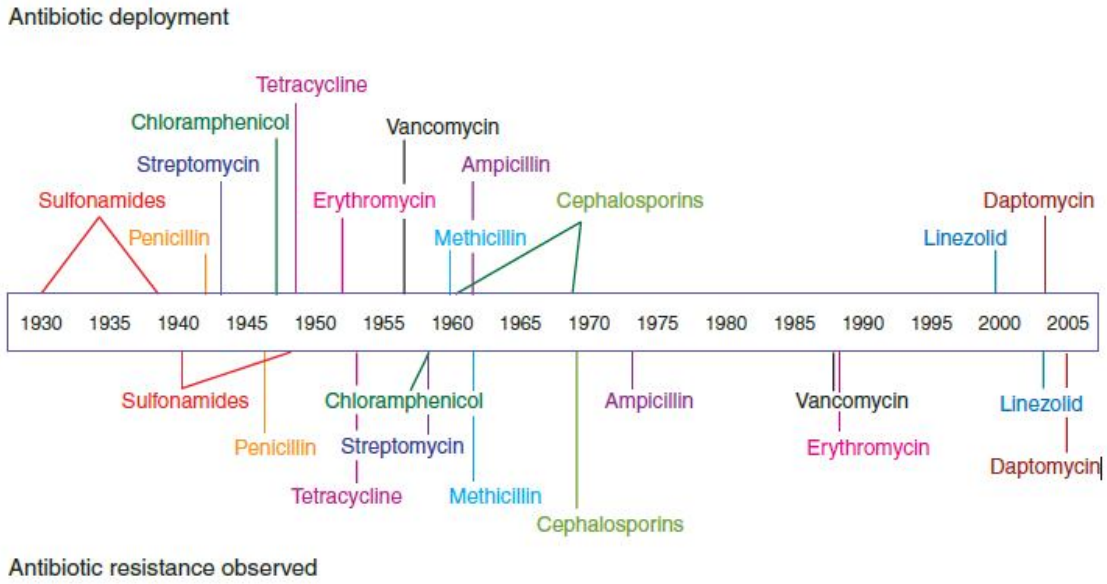

Figure 2 - Timeline of antibiotic deployment and the evolution of antibiotic resistance. (Clatworthy, Pierson et al. 2007)

The overuse of antibiotics has lead to the appearance of resistant bacteria, often the result of extensive selective pressure. Different mechanisms were developed by bacteria in order to escape new and improved antimicrobial agents:

- β-lactamase or penicillinase enzymes production, which act on the β-lactam ring, inactivating or destroying the antibiotic molecule;
- Incapacity of the antibiotic to break into the bacterial cell;
- Target alterations on the molecular basis, changing the inhibited metabolic pathway to an alternatively efficient one;
- Efflux pumps that reject the antibiotic before it can be effective.

The first line antibiotics used in hospital, like cephalosporins, are now being threatened by the emergence of resistance mechanisms like ESBLs. And the main challenge is now the rapid identification of the exact kind of resistance, in order to quickly apply the correct medication and avoid the increasing morbidity and mortality rates. These kind of strains often presents multidrug resistance, being

also resistant to amino-glycosides and fluoroquinolones, and thus reducing the therapeutic options for those severe infections. (Ghafourian, Sadeghifard et al. 2014)

1.4.1. β-lactams

For the past 60 years the β-lactams have been the most successful drug in the treatment of bacterial infections, for both Gram-positive and Gram-negative. In 2004, they represented over 65% of the worlds' antimicrobial market. Not surprisingly, the increased use of penicillins caused resistance to arise in the clinical setting. Penicillin G was the first β-lactam antibiotic to be introduced in clinic practice, in the early 1940s, and by 1944, the first reports of penicillin-resistant *Staphylococcus aureus* began to emerge. These were the first references to β-lactamase enzyme production, that inactivate the antibiotic molecule by hydrolyzing the β-lactam core. β-lactams act by mimicking the natural substrate of the Penicillin-Binding-Proteins (PBP) – D-Ala-D-Ala – which is responsible for cross-linking the peptidoglican section of the cell wall. By forming this complex, they inhibit the transpeptidation activity and disrupt the integrity of the cell wall, ultimately resulting in cell lysis. (Bush 2010; Bush and Jacoby 2010; Worthington and Melander 2013)

Non-β-lactams are usually useless against ESBL producers, because plasmids that carry the ESBL gene also carry genes encoding resistance to other classes of antibiotics, such as quinolones, aminoglycosides, and tripethoprim-sulfamethoxazole. (Delgado-Valverde, Sojo-Dorado et al. 2013)

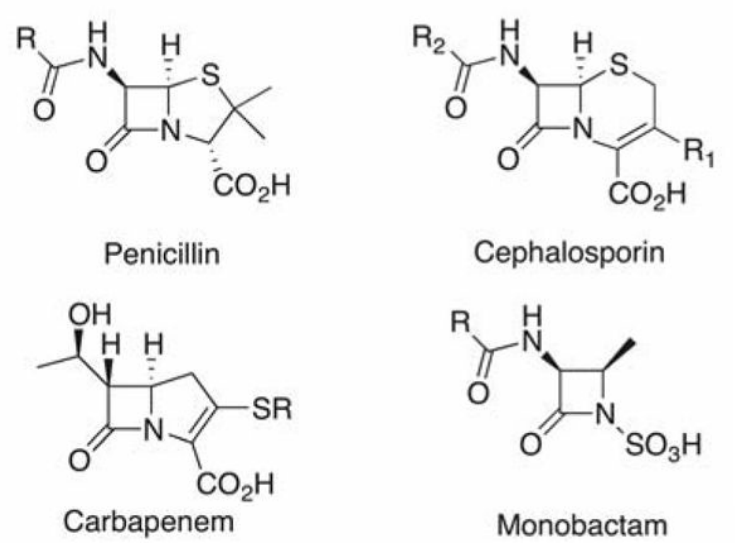

Figure 3 - Structures of the different β-lactam antibiotic classes. (Worthington and Melander 2013)

1.4.2 Mechanisms of resistance to β-lactams: β-lactamases

To escape β-lactam antibiotics effects, Gram-negative bacteria have developed multiple strategies: production of β-lactamases, novel PBPs with reduced affinity to β-lactams, porin mutations that reduce β-lactam entry or multidrug efflux pump that expel β-lactams out of cell. Of these mechanisms, β-lactamase production is still the most efficient. (Zeng and Lin 2013)

Clinically relevant β-lactamases include Extended Spectrum β-Lactamases (ESBL), AmpC β-lactamases, *Klebsiella pneumoniae* carbapenemases (KPC) and Metallo-β-lactamases. A combination of those can also be expressed, as well as nonenzyme resistance mechanisms, namely porin loss and efflux pumps. (Marsik and Nambiar 2011)

β-lactamase enzymes can be classified according to Amblers' molecular characterization system or the Bush functional classification scheme. The molecular classification is based on the amino acid sequence, and results in 4

groups of β-lactamases: A, B and D enzymes, which utilize serine for the hydrolytic activity, and class B metalloenzymes, which require divalent zinc ions to hydrolyze the substrate. As for the functional classification scheme, it has been updated from the original one, back in 1995, proposed by Bush *et al.*, and it takes in consideration the substrate and inhibitor profiles of the enzyme, sorting them in close correlation with the phenotypic characteristics. (Bush and Jacoby 2010)

1.5 Scope

The main goal of this thesis is to contribute to the knowledge of distribution and prevalence of resistance in selected species collected within the hospital environment. Microorganisms were isolated from samples, collected at the Centro Hospitalar do Baixo Vouga, EPE in Aveiro. The study focuses on antibiotic resistance strains, in resistant and MDR isolates. Since the community environment also seems to be an important reservoir of antibiotic resistant microorganisms, isolates were collected from patients attending the emergency room and were assumed to represent the community.

Being exposed the growing need for more accurate results, this thesis has 2 main goals:

a) the epidemiology of ESBL producing isolates in patients attending the emergency room (ER), diagnosed with an urinary tract infection (UTI) and older than 65 years old;

b) the epidemiology of AmpC producing isolates, in Centro Hospitalar do Baixo Vouga (CHBV)

2. Material and methods

2.1. Central Hospital characterization

The present work was performed in the section of Microbiology of the clinical pathology department, in the “Centro Hospitalar do Baixo Vouga, E.P.E. – Hospital Infante D. Pedro (HIP)”. HIP is one of the three units that compose the “Centro Hospitalar do Baixo Vouga (CHBV)” and includes several wards, namely internal medicine, general surgery, orthopedics, pediatrics, urology, infect logy, cardiology, pneumology, gynecology and obstetrics.

The emergency room of this hospital also comprises several medical specialties, in which clinical pathology is included.

The tests performed in the Microbiology Laboratory are of utmost importance to an accurate diagnosis allowing the identification of the pathogen and its resistance and/or susceptibilities to the antibiotics available, thus being essential for the success of patient treatment.

All the isolates used in this study were collected from both inpatients and patients attending the ER of “Centro Hospitalar do Baixo Vouga, E.P.E.”, from January to December 2014.

2.2. Bacterial strains

Isolates were collected according to the aim of each study (specified in chapters 1 and 2)

After entering the Microbiology laboratory, the sample was cultured in the appropriate culture medium. Urine samples were inoculated in CLED (Cystein Lactose Electrolyte Deficient medium) (BioMérieux, France), and incubated at 37ºC for 16 to 24 hours. If the culture presented $1x10^5$ CFU/ml or more, it was considered a significant bacteriuria and, thus, proceeded to isolation of the potentially pathogenic microorganism. All other types of samples (pus, sputum, blood) were inoculated in other culture medium: once positive for the presence of microorganisms, colonies were passed to MacConkey Agar (selective and

differential medium with violet crystal) (BioMérieux, France), Columbia Agar (with 5% sheep blood) (BioMérieux, France) or Chocolate Agar (obtained from Columbia Agar, by heating at 80ºC) (BioMérieux, France), depending on being Gram-negative bacilli or Gram-positive cocci, and if detection of hemolysis was needed.

Plates were then incubated for 16 to 24 hours at 37ºC, so that identification could be performed. Additional assays like Gram staining and biochemical tests were also made, in order to facilitate the choice of the panel to be used in the automated method.

Control strains were used as standard procedure in the Vitek2 system and also in the performance of phenotypic methods. The strains used were *E.coli* ATCC and *Serratia marcescens* as a negative and positive control for AmpC production. Three random *E. coli* reported as AmpC negative by the Vitek2® system were used to evaluate the specificity and sensitivity of both methods.

2.3. Identification and susceptibility testing (automated method)

Vitek2® (BioMérieux, France) is an automated system that allows the identification of the bacterial species and antibiotic susceptibility pattern of the microorganism in a 24 hour period. The system is based on an optic system combining a multichannel fluorometer and photometer, that register alterations in fluorescence, turbidity and color. (Figure 3, www.biomerieux.com)

The identification was made from a pure and fresh culture, and taking in consideration the results of a set of 64 tests, each containing an individual substrate, evaluating the metabolic activity of the organism: acidification, alcalinization, enzymatic hydrolysis or growth in the presence of inhibitors. Both contamination and alteration in oxygen levels were assured by a transparent film on all the wells of the card. Each card contains a bar code, reporting the type of ID card, lot number, expiring date and the corresponding sample identification for the equipment. In order to comprise all microorganisms, there are four distinct cards

available: 1) Gram-negative bacilli, fermenter or non-fermenter; 2) Gram-positive cocci and non-spore former bacilli; 3) yeasts; 4) Gram-positive spore-former cocci.

The fresh and pure culture was taken to a suspension of 0,5 to 0,63 McFarland and inoculated, trough a vacuum system, into the chosen card. The cards were then incubated at 35,5±1ºC and read every 15 minutes. The results are compared with a database of well characterized strains, and an ID is obtained with a certain degree of similarity of metabolic test.

According to CLSI criteria, the results are only acceptable when the equipment is verified by the quality control system. For that, reference strains are tested, *E. coli* ATCC 25922 and *K. pneumonia* ATCC 700603, and only then results are validated.

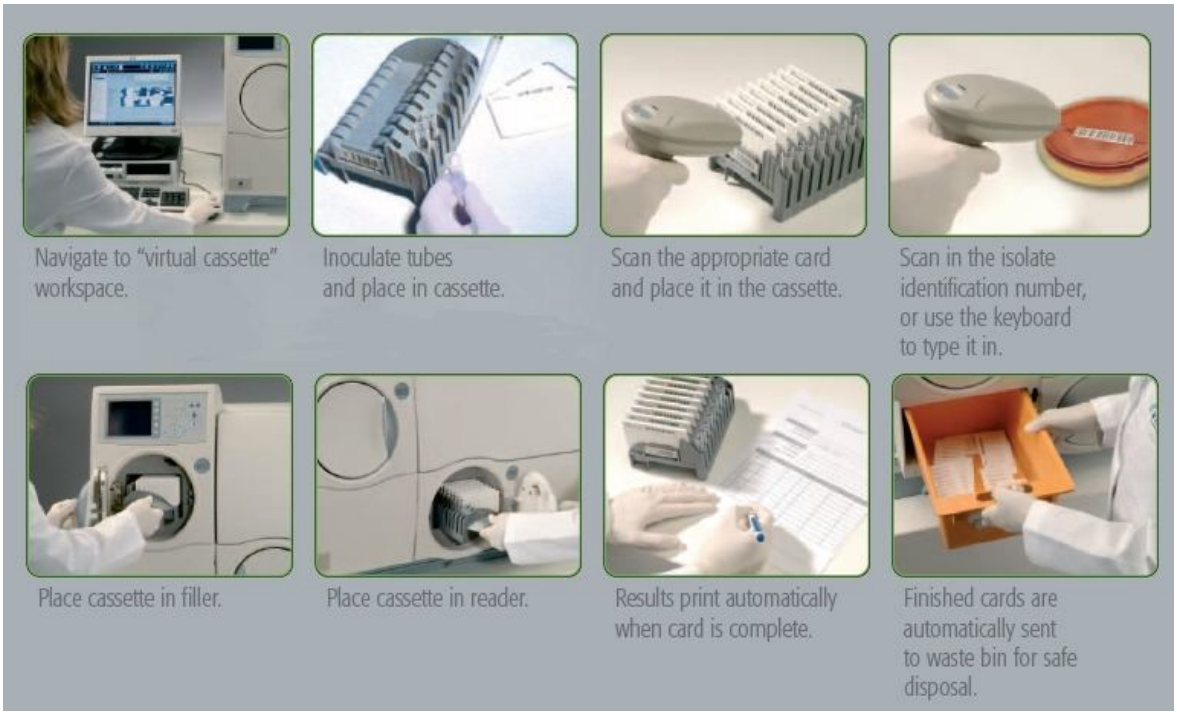

Figure 4 - Vitek2® automated system schematic work flow (www.biomerieux.com)

In a similar methodology, Vitek2® is also capable of testing the susceptibility to a battery of antimicrobial agents, by comparing MIC patterns. The type of card is chosen according to the Gram staining, and can vary between AST-N222 and AST-N192 cards (for Gram-negatives), AST-619, AST-586 e AST- ST01 (for Gram-positives) and YST ID for yeasts. The results are expressed in sensitive, intermediate or resistant phenotype.

2.4 Phenotypic methods

2.4.1 Quantitative method Etest

Strains identified by the automated system as ESBL producers were confirmed using phenotypic tests in order to detect the production of β-lactamases. A fresh and pure culture is taken to a suspension of 0,5 McFarland and spread into a Mueller Hinton Agar plate, in all directions, with a sterile swab. After being left to dry for five minutes, Etest strips (AB BioMérieux, Sweden) were placed on top of the medium.

Each Etest strip to detect ESBL producers is divided in two regions: in one side, a crescent concentration gradient of one antibiotic, from the center to the top, and in the other side, the same antibiotic in association with a β-lactamase inhibitor. The strips contain a cephalosporin and an association of a cephalosporin with clavulanic acid as follows: cefotaxime and cefotaxime + 4 µg/ml clavulanic acid (CT/CTL), ceftazidime and ceftazidime + 4 µg/ml clavulanic acid (TZ/TZL) and cefepime and cefepime + 4 µg/ml clavulanic acid (PM/PML)

The Etest strips used to detect ESBL producers were as explained in Figure 4. The plate was incubated for 24h, at 37ºC and the interpretation of the ellipses formed was interpreted.

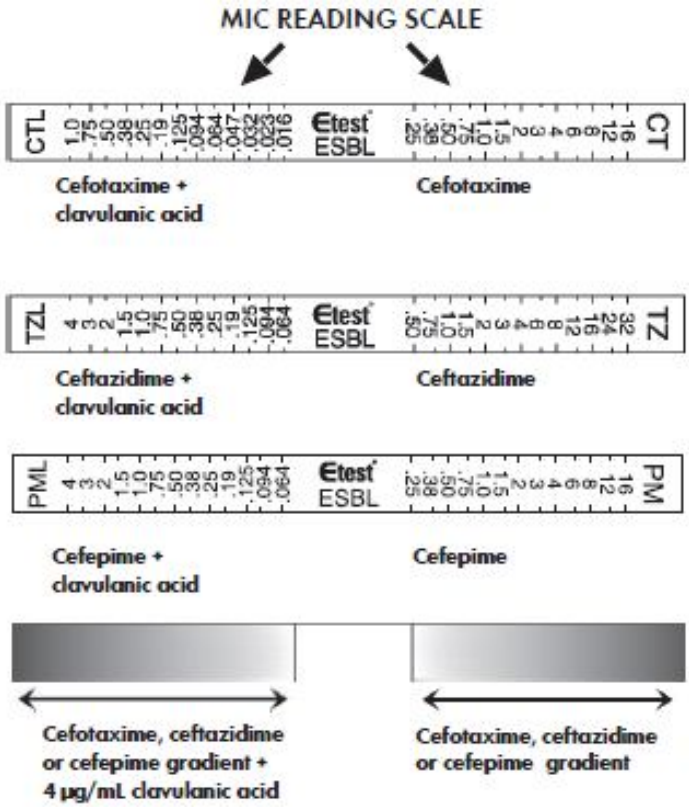

Figure 5 - Configuration of Etest ESBL strips (www.abbiodisk.com)

A positive result is obtained when the ratio between the value of the MIC obtained for the antibiotic alone and its association with an inhibitor, for example, CT/CTL, is higher than eight. On the other hand, if the result obtained is inferior to eight, the result is considered negative. If it is not possible to obtain a MIC value either for the antibiotic alone or the antibiotic in combination with the inhibitor the result is considered non determined.

2.4.2 Cefoxitin disc test

Since resistance to cefoxitin is a good indicator of the presence of plasmidic AmpC, cefoxitin disc test by diffusion in an agar plate was used to determine the susceptibility in all the isolates suspected to be an AmpC producer.

A pure and fresh culture was used to prepare a 0,5 McFarland that was spread on a Mueller-Hinton agar plate, in all directions, with a sterile swab. A

cefoxitin disc (30µg) was placed on the surface and plate incubated at 35±2ºC for 16-18h, in aerobic conditions. The result was read in the diameter of the inhibition zone: the microorganism is considered susceptible to cefoxitin if the inhibition diameter is larger than 18mm; cefoxitin resistant strains have 17mm or less of inhibition halo diameter and are, therefore, potentially AmpC producers.

3. Chapter 1- Epidemiology of ESBL-producing Isolates causing UTI in the elderly (2013-2014)

3.1. Introduction

The world population is getting older and therefore more prone to acquire infections than can lead to hospitalization, and in a more complicated scenario can cause severe morbidity and mortality. Among those infections, urinary tract infections (UTIs) are one of the most prevalent community-acquired infections. Patients with severe illnesses and/or hospitalized for long periods are the ones showing higher risk of being infected with ESBL producers because of the inherent use of large amounts of antibiotics. Among the hospital environment, transmission usually occurs by fecal-oral way, and is mostly related to hand contact between healthcare workers. (Ghafourian, Sadeghifard et al. 2014)

Extended spectrum β-lactamases are capable of hydrolyzing various classes of β-lactam antibiotics: penicillins (e.g. ampicillin and piperacillin), cephalosporins of the first (cephalotin), second (cefuroxime), third (ceftazidime and cefotaxime) and fourth (cefepime) generations, and monobactams (aztreonam), but not carbapenems. Therefore ESBL producing strains are able to hydrolyze all the β-lactam antibiotics with high efficacy, except for carbapenems. (Livermore and Brown 2001) There are known and described more than 1000 distinct β-lactamases. On the other hand, ESBLs can be inhibited by β-lactamase inhibitors like clavulanic acid, tazobactam or sulbactam. This phenotypic characteristic allows to distinguishing this kind of strains, except for the presence of a chromosomal AmpC, which makes them resistant to inactivation by a beta-lactamase inhibitor. (Perez, Endimiani et al. 2007) Most ESBL mechanisms are mutational derivates of the classical TEM-1, TEM-2 and SHV-1 enzymes, resulting of one to four amino acid changes. These changings allow attack on aminothiazolyl cephalosporins and monobactams. (Livermore and Yuan 1996)

Epidemiological studies, such as SMART (Study for Monitoring Antimicrobial Resistance Trends), concluded on the prevalence of cephalosporinases mediated by ESBL in *E. coli* (17,6%) and *K. pneumonia* (38,9%) in Europe; Significantly

lower were the results for the United States, 8,5% and 8,8%, respectively, and significantly higher, in Asia, with 40,8% for *E. coli* and 21,5% for *K. pneumoniae*. (Delgado-Valverde, Sojo-Dorado et al. 2013)

3.2. Results and Discussion

3.2.1. Characterization of the collected samples

A total of 1263 isolates were recovered during the timeframe of this study, January 2013 to December 2014, at Centro Hospitalar do Baixo Vouga, EPE, Portugal. The Centro Hospitalar do Baixo Vouga, EPE, comprises three hospitalar units, namely Hospital Infante D. Pedro, Hospital de Águeda and Hospital Visconde de Salreu. The population analyzed was non-repetitive and only single isolate/patient was included in the study. The isolates were selected according to the following criteria to each patient: primary diagnosed with an UTI, older than 65 years old and attending to the ER. As the infection under study was an UTI, the biological product selected was urine. Figure 5 shows the distribution of the samples by the different methods used in sample collection, namely, midstream collection and civet (catheter).

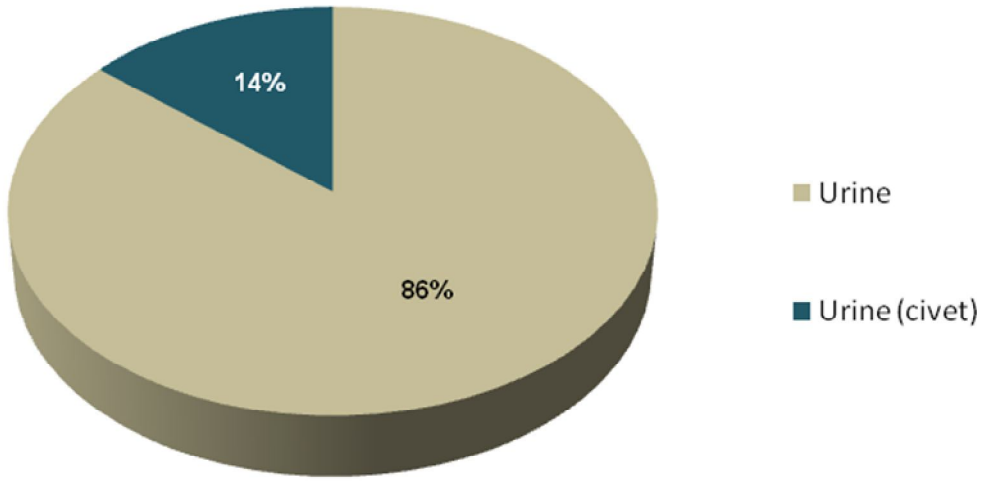

Figure 6 - Distribution of the samples by the different methods used in sample collection.

The majority of the samples was collected by the midstream method, nevertheless 14% of the samples were retrieved from civets. The use of civets in patients older than 65 years old, naturally increases since they can become more dependent. Despite the percentage of samples retrieved from civets being low this value requires vigilance, since the patients with civets are usually in higher risk of infection leading to an increase of hospitalization time.

Figure 6 shows the analysis of the distribution of the samples collected by gender. It was observed, as expected, that 60% of the strains were isolated from urine samples retrieved from women, whereas 40% were retrieved from men. This result can be explained by the anatomic differences of the urinary system between men and women, which makes the UTIs more common in women than in men. On the other hand, in an era where women live longer than men it is expected to have women more represented than men.

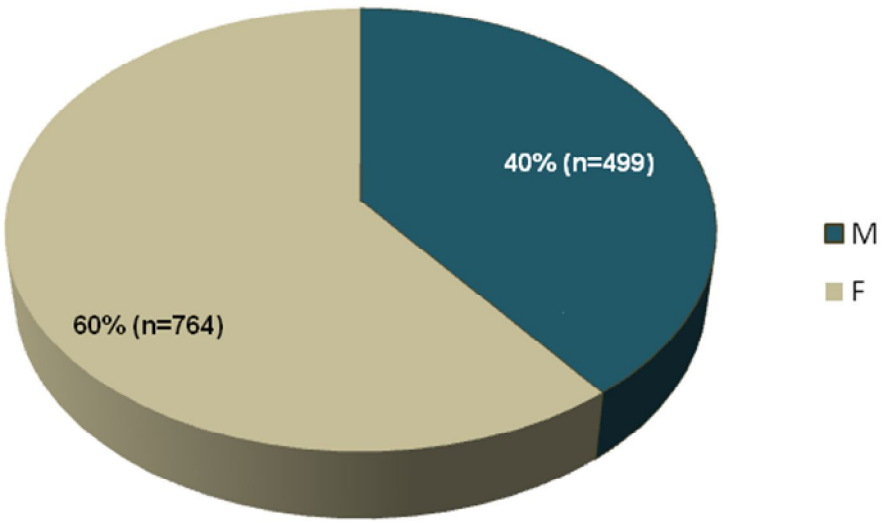

Figure 7 - Urinary tract infections distributed by gender; M- male, F- Female.

3.2.2. Characterization of the agents causing UTI

As mentioned above the isolates were all collected from urine. Figure 8 shows the general distribution of the isolates based in the Gram staining technique. It is shown that the vast majority of the isolates, 86%, were Gram-negative, whereas the Gram-positive corresponded to 8% and yeast to 6%. These results are in accordance with the in the literature.

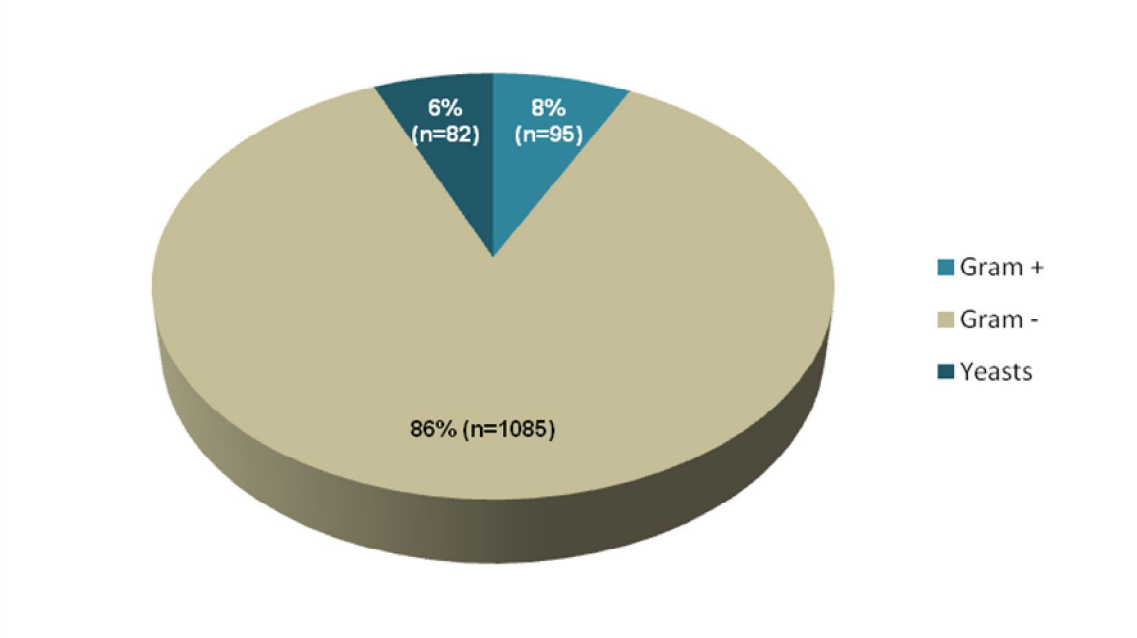

Figure 8 - Distribution of the UTIs causing agents according to Gram staining.

Also, it has been reported in previous studies that the most frequent bacteria isolated from urine, in patients with an UTI, belong to the *Enterobacteriaceae* family. In the present study similar results were obtained, as it can be observed in Figure 9. The samples analyzed showed a high phylogenetic diversity being *Enterobacteriaceae* the more represented family with 76% of the isolates, whereas other isolates than those belonging to the *Enterobacteriaceae* family represented 24% of the total isolates.

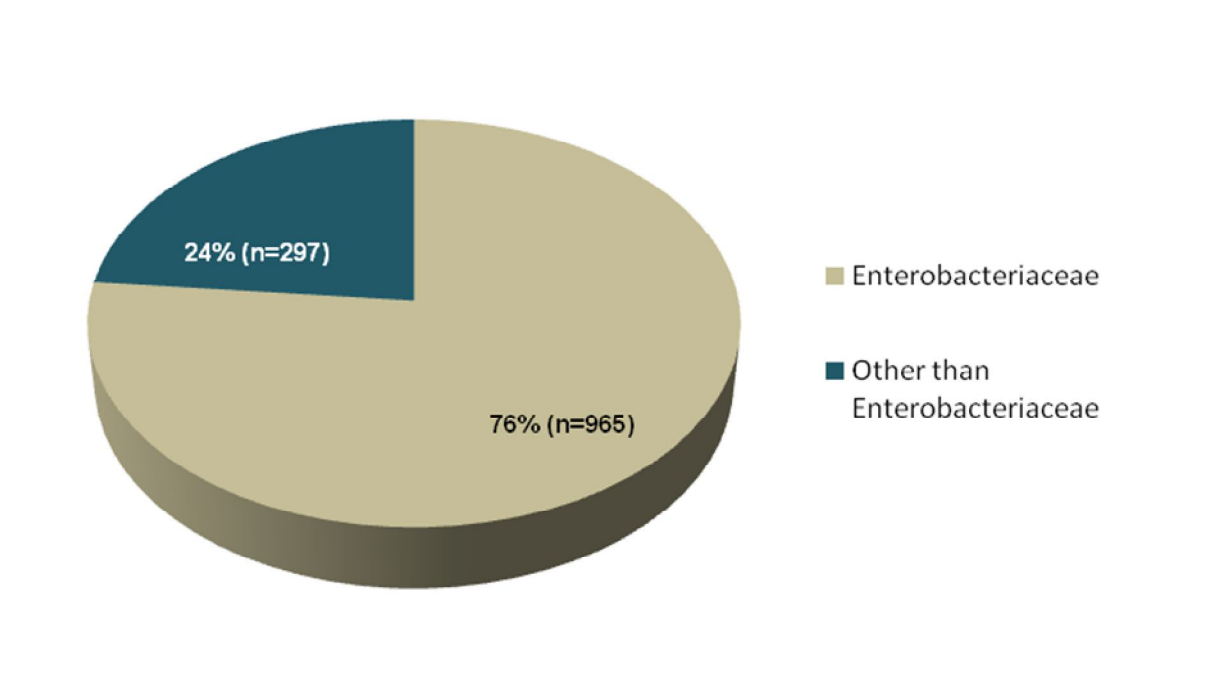

Figure 9 - Classification of the agents causing UTIs.

Among the *Enterobacteriaceae* family the most prevalent microorganisms isolated were *E. coli* (n=585)*, K. pneumoniae* (n=203)*, Proteus mirabilis* (n=82) as it is shown in Figure 10*.* Regarding the microorganisms classified as "Other than *Enterobacteriaceae*", *Enteroccocus spp, Pseudomonas aeruginosa* and *Candida spp*, were the most common also isolated. The results obtained in this study are in accordance with the literature which refers *E. coli* as the prevalent species causing UTI. During two years, Correia, Costa et al. (2007) studied the etiology of urinary tract infections in the Centro Hospitalar do Nordeste, E.P.E. - Unidade Hospitalar de Bragança. In the population studied, the prevalent species isolated were *E. coli,* followed *K. pneumoniae. (Correia, Costa et al. 2007)* The Study for Monitoring Antimicrobial Resistance Trends (SMART) has been monitoring susceptibilities of Gram-negative pathogens, since 2009, in a study involving several hospitals representing the North, Center and South of Portugal. During 2009 and 2010, a total of 249 isolates were collected from patients diagnosed with an UTI and the prevalent species isolated was *E. coli*, representing 48.2% of the isolates, followed by *K. pneumoniae* representing 18.5% of the total isolates. (Ferreira, Diogo et al. 2012)

Almost the same period, Ferreira, Diaz et al. (2012) also studied the prevalence of pathogens causing UTIs in patients older than 65 years old attending the ER of the Hospital Infante D. Pedro E.P.E. Interestingly, in this case the prevalent species identified was *K. pneumoniae* followed by *E. coli.* However, more recently Magalhães, Brandão et al. (2014) reported that the percentage of *E. coli* isolates has been increasing since 2011, comparing with the percentage of *K. pneumoniae* isolates, which in 2011 was higher than *E. coli* but has been significantly decreasing in the last two years. (Ferreira, Diaz et al. 2012; Magalhaes, Brandao et al. 2014)

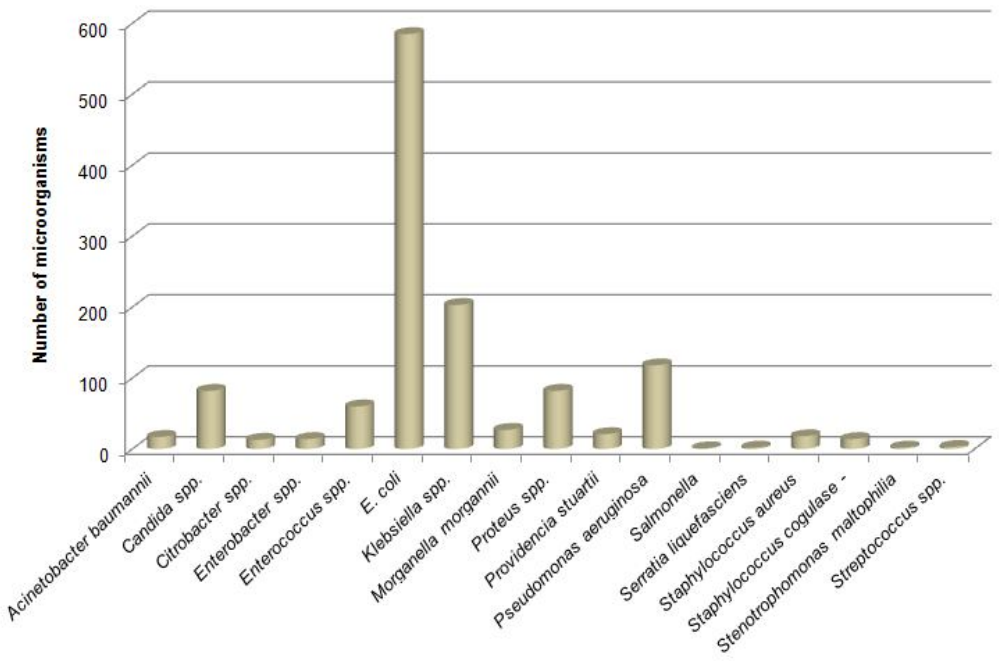

Figure 10 - Number of microorganisms isolated from the samples collected, causing UTIs.

Among *E. coli* and *K. pneumoniae* further analysis was performed regarding ESBL production. ESBL- producers were selected by indication of the automated method Vitek2® and confirmed by Etest phenotypic method. There were no significant discrepancies between both methods.

In the last two years *E. coli* was the organism causing higher number of UTI, representing 46% of the cases both in 2013 and 2014. However, the percentage of *K. pneumoniae* ESBL-producers, 35% and 37%, was always higher than *E. coli* ESBL- producers which had a percentage of 13% in 2013 and 19% in 2014 (Table 1). Worth noticing is the increase of ESBL- producers in both species.

Table 1 - Number of isolates selected, percentage of *K. pneumoniae* and *E.coli* identified, and percentage of ESBL producers respectively

Year	Total number of clinical isolates	% *K. pneumoniae*	% *E. coli*	% *K. pneumoniae* ESBL	% *E. coli* ESBL
2013	614	18%	46%	35%	13%
2014	649	14%	46%	37%	19%

However, a study of Roxo, Magalhães *et al*. (2015) on the epidemiology of ESBL-producing isolates causing UTI in the elderly, in Aveiro, shows a reduction in the percentage of ESBL-producers until 2013. In 2011, 82,5% of *K. pneumoniae* isolates were ESBL-producers, and it progressively decreased until 2013. Nevertheless in 2014, the percentage slightly increased to 37%. *E. coli* ESBL-producers have also progressively diminished until 2013, exhibiting an increase (19%) in 2014. The high number of ESBL-producers in this type of population is disturbing and requires attention. A possible explanation is the constant in and out flux of elderly people living in nursing homes or their own house, that repeatedly visit the hospital and therefore can represent a reservoir and/or a vehicle of ESBL-producers.

3.2.3. Patients provenance and outcomes

Analyzing the data in order to conclude on the provenance of the patients carrying an ESBL-producer causing an UTI, it was not possible to obtain an accurate information in 50% of the cases due to the lack of information in the data base consulted. Nevertheless, it was possible to conclude that 34% of the patients carrying an ESBL-producer were provenant from home, whereas 17% were coming from nursing homes (Figure 11). Despite of the lack of information regarding 50% of the cases, considering the criteria used to select the isolates we can conclude that they are disseminated in the community. Community-acquired strains possessing ESBLs might be selected from the existing gastrointestinal flora when it is exposed to broad-spectrum antimicrobial agents. Thus, ESBL producers are expected to be present in general practice, but their occurrence has rarely been reported, probably because screening for ESBLs requires special tests and operator expertise. (Arpin, Dubois *et al.* 2003)

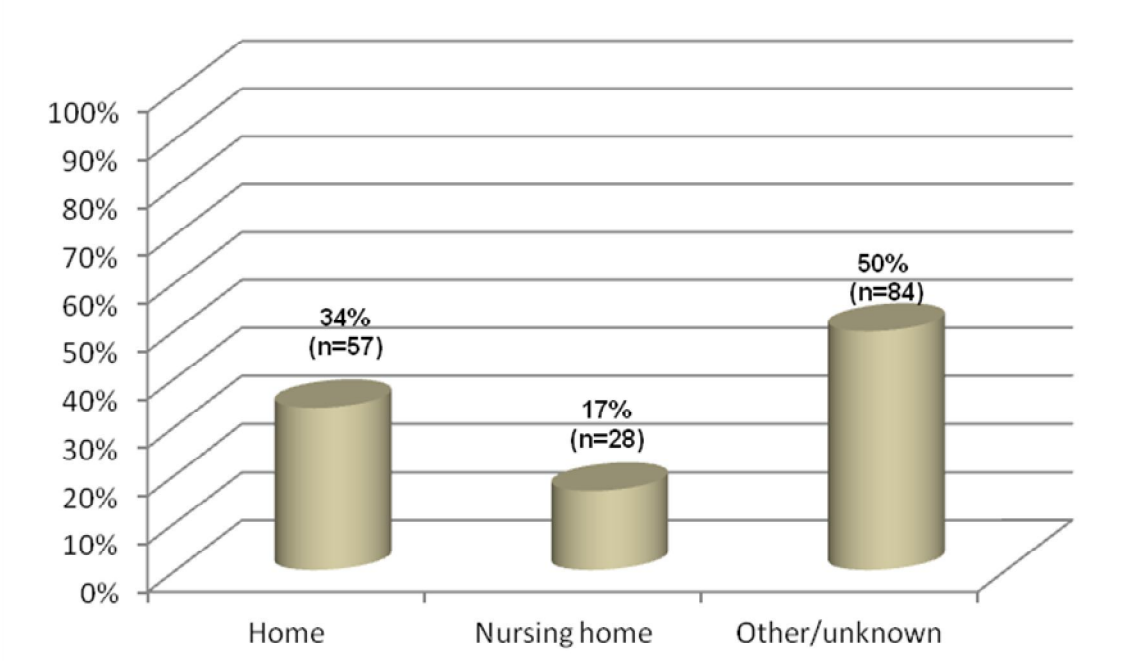

Figure 11 - Provenance of the patients carrying an ESBL-producer causing an UTI.

Several studies report the difficulties in the treatment of infections caused by ESBL-producers. (MacVane, Tuttle *et al.* 2014; Cho, Jung *et al.* 2015; Osthoff, McGuinness *et al.* 2015). By reviewing the cases of UTIs caused by multidrug resistant strains, MacVane (2014) concluded that a history of recurrent UTIs, recent treatment with antibiotics, recent hospitalization, and previous isolation of an ESBL-producing microorganism, when compared with controls, were the main risk factors. Plus, infection-related mortality of 7.2% and 30-day UTI readmission were higher in ESBL positive cases than in control patients, which had around 2% mortality rates. (MacVane, Tuttle et al. 2014) Also, in an Australian study by Osthoff (2015), the length of previous stays in the hospital prior to the urine sampling episode, and residency in a nursing home were also found to raise the possibilities of having an UTI caused by ESBL positive bacteria. (Osthoff, McGuinness et al. 2015) In our case, and in accordance with those previous studies, the mortality rate was around 11% (Figure 12). This is a relatively low number, despite being evaluating elderly patients with infections caused by multiresistant bacteria. The timely detection of these strains, together with an appropriate initial antibiotherapy may minimize their impact on outcomes of patients with UTI.

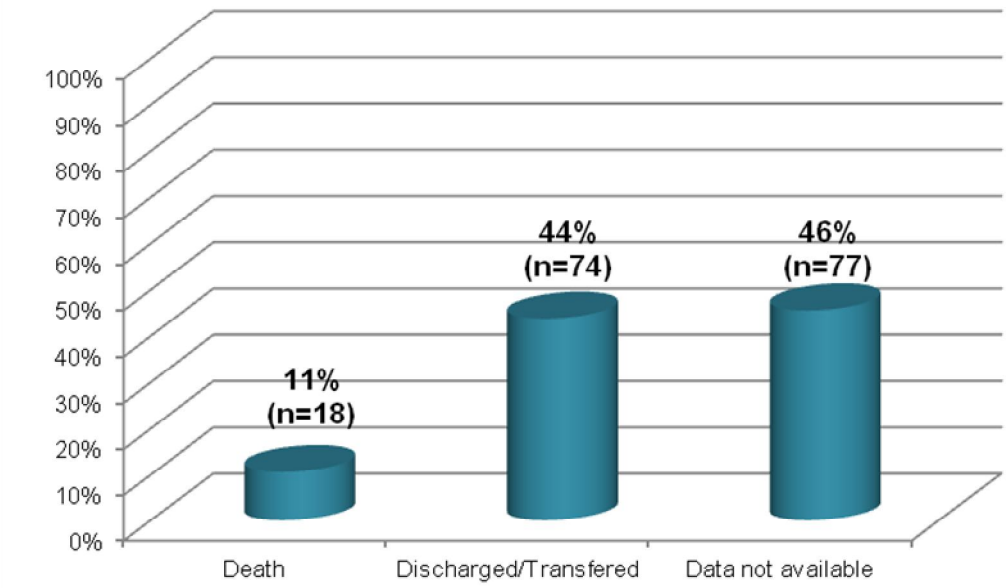

Figure 12 - Type of outcome of the patients carrying an ESBL-producer causing an UTI

3.3. General conclusion

This study reveals disturbingly high numbers of ESBL-producing strains detected in patients over 65 years old with UTIs. Motive of especial concern is the fact that these samples were retrieved from outpatients, which makes this kind of resistance not only confined to hospital environments anymore, but already spread in the community. Repeatedly visiting the hospital, or living in nursing homes, turns these already fragile patients into potential reservoirs of these ESBL-producing strains.

It becomes clear that timely use of the correct antibiotics is critical in minimizing the transmission and even deaths caused by ESBL-producing strains.

4. Chapter 2- Epidemiology of AmpC-producing *Enterobacteriaceae*

4.1. Introduction

The first enzyme reported to destroy penicillin was an AmpC β-lactamase of *Escherichia coli*, in 1940, although not named AmpC yet, and the sequence of this gene was much later reported – in 1981. (Jacoby 2009) This kind of resistance mechanism can be found in *Pseudomonas aeruginosa* and in many *Enterobacteriaceae* species, such as *Klebsiella spp*., *Salmonella spp*., *C. freundii*, *E. aerogenes*, *P. mirabilis*, and *E. coli. (Thomson 2010)* According to their amino-acid sequence, AmpC enzymes belong to class C of Ambler, whereas in the functional aspect, they are assigned to group 1 of Bush-Jacoby classification. (Marsik and Nambiar 2011)

Plasmid-borne AmpC cephalosporinases were first detected in late 1980s, and appeared to be genetic descendants of the chromosomally encoded AmpC enzymes. The presence of these genes on plasmids made its transmission easier, and its over expression yields clinical resistance of the strains to almost all β-lactams: narrow and broad spectrum cephalosporins, β-lactam/ β-lactamase inhibitor combinations and aztreonam. (Gupta, Tak *et al.* 2014; Helmy and Wasfi 2014) AmpC genes in the chromosome produce low level of β-lactamase expression, because they are normally repressed. But they can be de-repressed, induced by antimicrobials such as cefoxitin, and thus, becoming hyper expressed. On the other hand, AmpC genes located in plasmids constitutively express the β-lactamase. (Marsik and Nambiar 2011) High level producers typically present cefoxitin-resistance. AmpC enzymes are not active against cefepime, cefpirome and carbapenems, thus being sensitive to those agents. Strains carrying this type of enzyme are therefore sensitive to cefepime (a fourth generation cephalosporin). But when AmpC is combined with other type of resistance mechanism, such as porin loss or efflux pumps, resistance levels increase significantly. (Marsik and Nambiar 2011) Meropenem remain the best treatment option in treating infections caused by this kind of pAmpC producers, even in case of co-production of ESBL enzymes. (Helmy and Wasfi 2014)

4.2. Results and discussion

4.2.1. Characterization of the collected samples

A total of 62 isolates were collected during the timeframe of this study, January 2014 to December 2014, at Centro Hospitalar do Baixo Vouga, EPE, Portugal. The Centro Hospitalar do Baixo Vouga, EPE, comprises three hospitalar units, namely Hospital Infante D. Pedro, Hospital de Águeda and Hospital Visconde de Salreu. The population analyzed was selected based on the suspicion of AmpC production given by the Vitek2® automatic system and confirmed by cefoxitin disc phenotypic test.

Analyzing the distribution of the isolates by gender it was observed that 63% of the isolates were collected from samples retrieved from male patients and 37% were collected from samples retrieved from female patients (Figure 13).

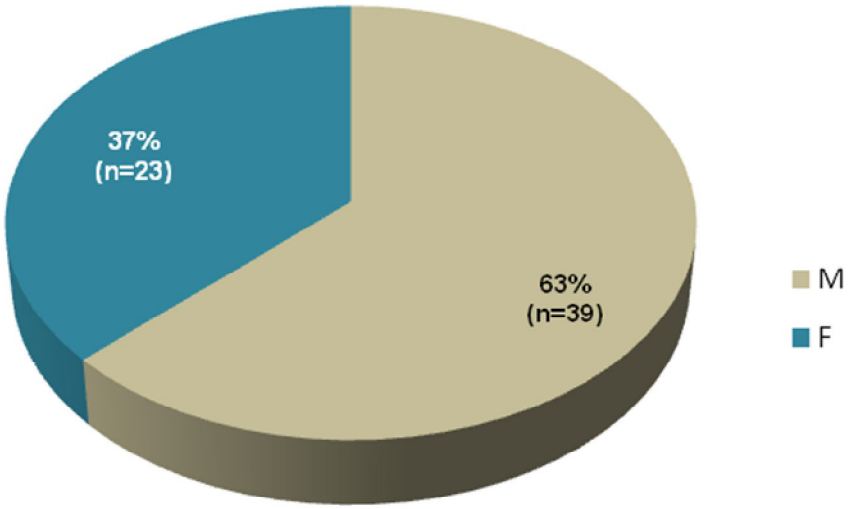

Figure 13 – Distribution of AmpC positive samples by gender; M – male, F – female.

Assuming that these results reflect the distribution and characterization of the AmpC-producers isolated from patients using the Centro Hospitalar do Baixo Vouga, E.P.E., it can be concluded that AmpC-producers are more common among male patients rather than female. Interestingly, in a study from (Brandão 2013), were phenotypic methods to detect AmpC-producing *Enterobacteriaceae* were evaluated, in the same hospital, reports that this type of isolates were more common in women. The authors justify the results obtained due to the fact of the majority of the isolates had been collected from a sample urine retrieved from patients diagnosed with an UTI, which is known to be more common in women rather than men, due their anatomic differences. (Brandão 2013) In the present study 52% of the isolates were also isolated from samples collected from urine, however in this case most of them were retrieved from men. The remain isolates were isolated from pus samples, 35% and 10% were distributed among other kinds of sample, such as sputum, blood or catheter tips (Figure 14).

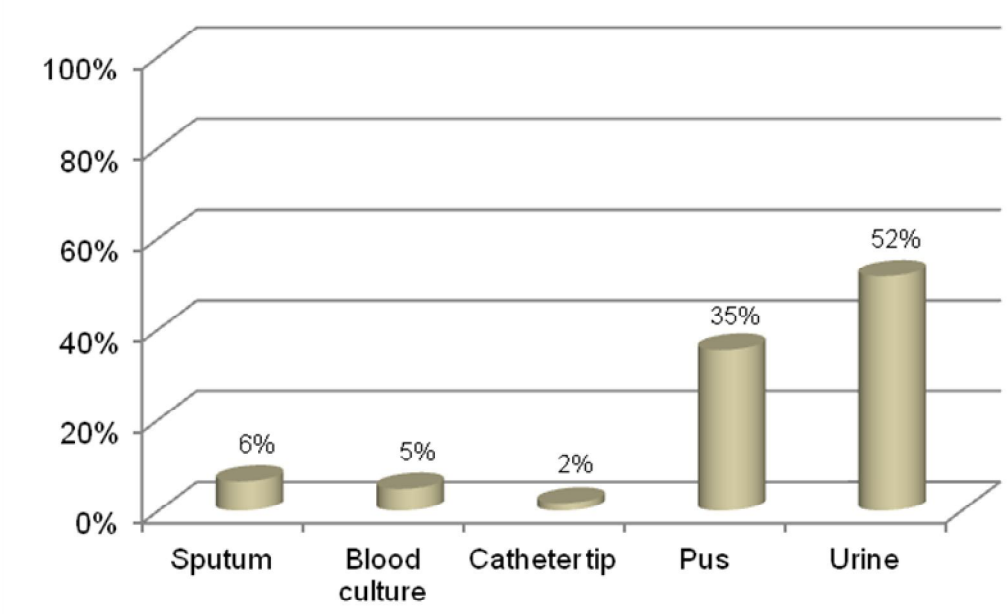

Figure 14 - Type of sample from which AmpC positive isolates were isolated.

Worth noticing the percentage of isolates collected from pus samples. Despite MRSA being the major infection agent causing skin and soft tissue infections occurring in diabetics and in elderly, and thus making these infections difficult to treat, the presence of AmpC-producers makes the scenario even worse, limiting therapeutic choices. Considering the provenance of the patients, they were classified as inpatients and outpatients, if they were staying in the hospital or if they were only attending the ER at the moment of the sample collection, respectively. From a total of 62 isolates, 60% were isolated from samples collected from inpatients in different services at CHBV, while 40% were isolated from samples from patients attending to the ER (Figure 15).

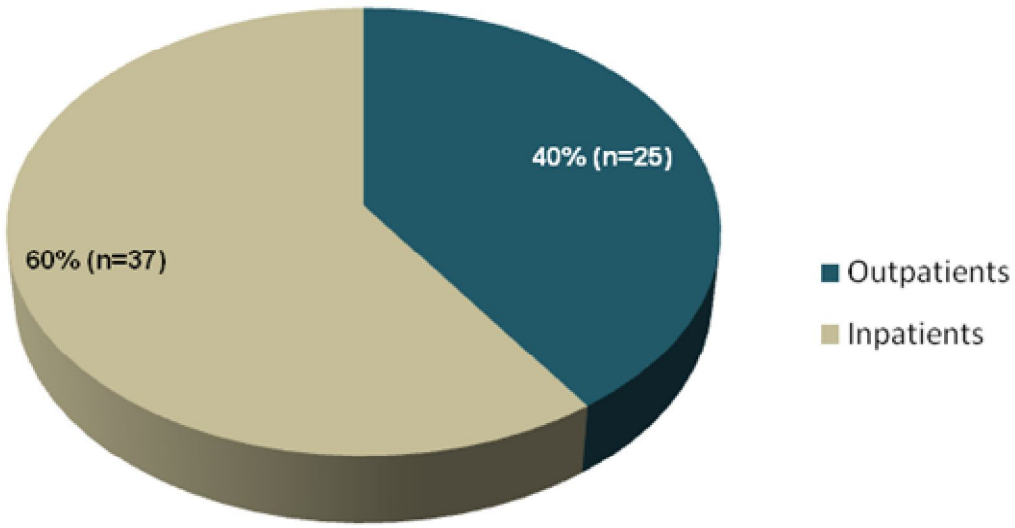

Figure 15 - Distribution of the isolates considering if they were collected from inpatients or outpatients.

According to the results obtained by (Brandão 2013) it is possible to conclude that there is a shift in the distribution of these type of isolates in the population served by CHBV, since in their study AmpC-producers were more prevalent in outpatients. Nevertheless, the results obtained in the present study show a considerable high prevalence of this kind of resistance spread in the community, which can be explained by the difficulties in detecting AmpC enzymes, as well as wrong therapeutical treatment, leading them to have successfully spread throughout the community. (Maraskolhe, Deotale *et al.* 2014)

4.2.2. Characterization of the AmpC-producing isolates

Among the 62 isolates recovered, the automatic system VITEK2® identified 50% of the isolates belonging to *Morganella morgannii* species followed by *E. coli,* 29% and *E. aerogenes,* 13%, being those the more representative species (Figure 16).

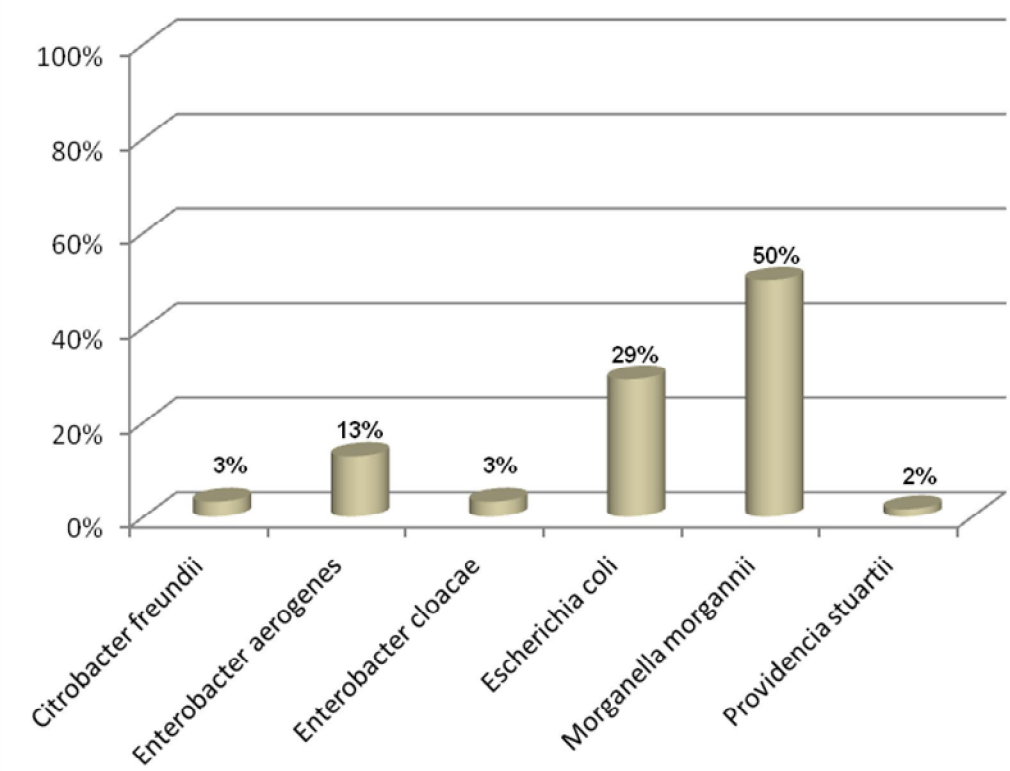

Figure 16 - Distribution of different causing agents among all isolates tested positive for AmpC expression

Portuguese and Indian studies report *E. coli* and *K. pneumoniae* as the more frequent species as AmpC-producers (Manageiro, Ferreira *et al.* 2010; Maraskolhe, Deotale *et al.* 2014), however that was not the case in the present study. Morganella morgannii was the most frequent species. In fact, its percentage was higher than expected, since the most common biological product studied was urine and *M. morgannii* species is not the most common pathogen reported to cause urinary tract infections. In fact, the majority of *M. morgannii* isolates were found to be recovered from pus samples. The results obtained are in accordance with those obtained by Brandão (2013) in the same hospital, were despite *M. morgannii* species was not the most prevalent, it was already the second more represented species. (Brandão 2013)

AmpC detection is supposable unnecessary in organisms that produce inducible chromosomal AmpC, being the species identification the only one needed to make it indicative – *E. cloacae*, *E. aerogenes*, *C. freundii*, *S. marcescens*, *Providencia* sp., *Morganella morgannii*, *Hafnia alvei*, *Aeromonas* sp., and *P. aeruginosa*. The results obtained in the present study are worrisome since the species isolated are reported to possess an inducible chromosomal AmpC and these microorganisms have the ability to develop resistance during antimicrobial therapy. (Thomson 2010) Also, reports of multiple β-lactamases in a single pathogen, generally leading to AmpC masking the effect of ESBLs, are increasing. These facts improve the possibility of mistakenly reporting them as ESBL negative and thus being wrongly medicated. Organisms producing high levels of AmpC will typically give a positive ESBL screening, but will not show the typical increased sensitivity with clavulanic acid. (Jacoby 2009)

In the present study, the carriage of an AmpC alone and both an AmpC and an ESBL was analyzed. Fifty two per cent of the isolates carried an AmpC alone, followed by 45% of isolates carrying a combination of an AmpC with an ESBL (Figure 17). A study of (Rawat, Singhai *et al.* 2013) showed ESBL production was seen in 39% isolates (43.7% of isolates of *Enterobacteriaceae* and 34% isolates

Pseudomonas spp.). Among *Enterobacteriaceae*, 18.7% were pure ESBL and 25% were ESBL and AmpC co-producers. (Rawat, Singhai *et al.* 2013) Another report from Singh, Shahid *et al*. (2011) shows that out of 49 ESBL positive isolates, co-existence of other *bla* genes with bla_{ampC} was present in 57.1% (28/49) isolates of *Enterobacteriaceae.* (Singh, Shahid *et al.* 2011)

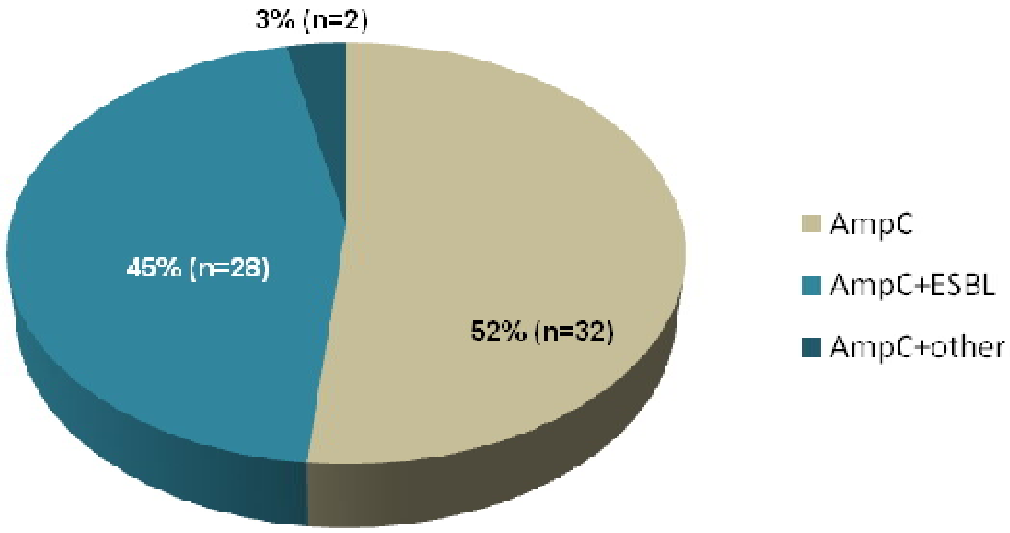

Figure 17 - Distribution of combined resistance mechanisms among AmpC positive strains.

Accurate data on AmpC testing should be possible, although they are less common than ESBLs, because they have been associated with false cephalosporin, and sometimes falsely reported as ESBL negative. (Thomson 2010) However, these isolates exhibited a multidrug resistance phenotype, therefore these findings highlight the importance of routine detection of these mechanisms. The majority of pAmpC producers is not susceptible to multiple antimicrobial substances including β-lactamase inhibitors such as clavulanic acid, in contrary to ESBL producers. The susceptibilities for cefepime and carbapenems are usually not affected by pAmpC β-lactamases. (Conen, Frei *et al.* 2015) The combination of the automated method with the cefoxitin disc seemed to be a

reliable choice for this detection (Figure 18). The use of the Etest strips for the detection of AmpC was misleading since a high number of undetermined results were obtained. These findings highlight the importance of routine detection of these mechanisms.

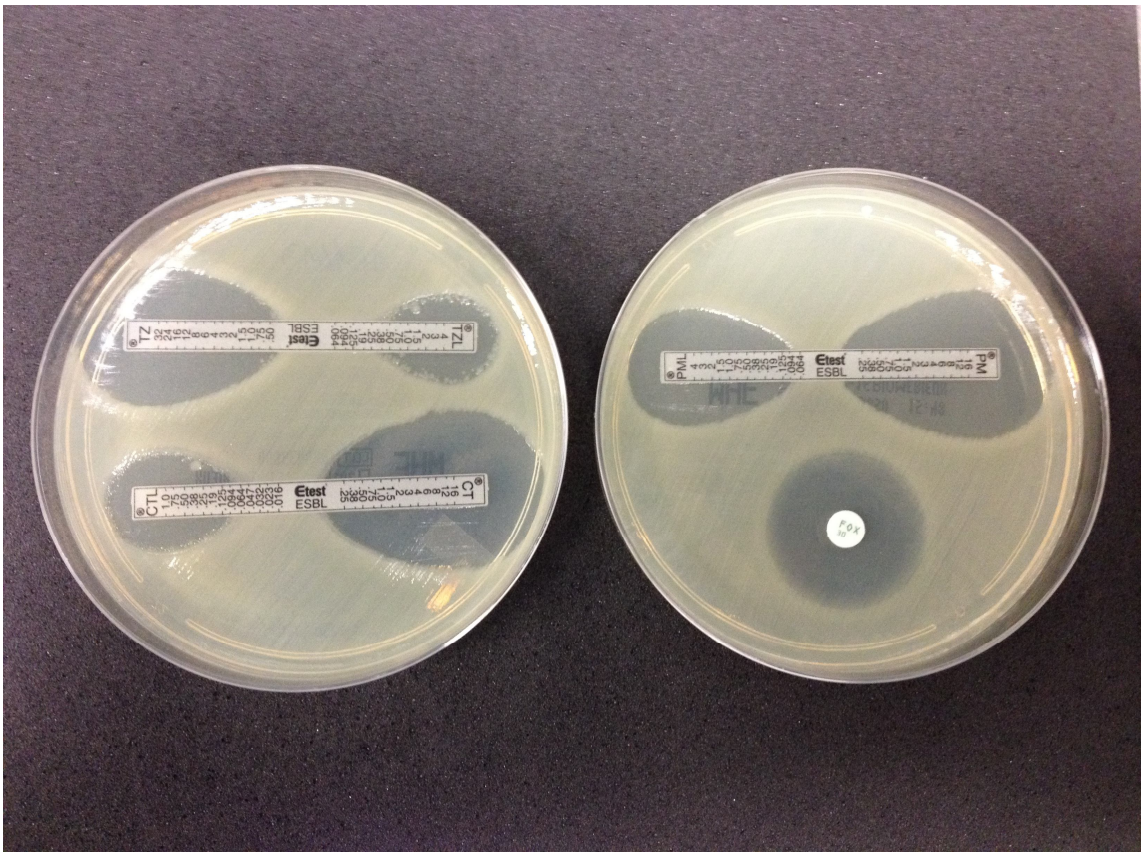

Figure 18 - Muller-Hinton agar (BioMérieux, France) showing an E-test combined with cefoxitin disc for the detection of AmpC.

Exception made to non-lactose fermenting Gram-negative bacteria intrinsically resistant to cephamicins, resistance to cefoxitin is suggestive of an AmpC enzyme. But it is not clear and specific, since other strains can express this resistance: carbapenemase producers some class A β-lactamases and decreased levels of outer membrane porin production (in *K. pneumonia* and *E. coli*). (Jacoby 2009) pAmpC are known to exist in various species lacking inducible chromosomal AmpC (cAmpC) genes including *Klebsiella spp*., *Proteus mirabilis*, *Salmonella enterica* and *Shigella spp*.

Some authors report that empiric antimicrobial therapy was inappropriate in more than 30% of the cases of an infection caused by a pathogen identified to be an AmpC-producer. Therefore, rapid identification of pAmpC carriers is needed and new microbiological methods are required to simplify rapid and reliable detection of pAmpC carriers. (Conen, Frei *et al.* 2015) According to the results obtained, in the present study, 71% of the patients carrying an isolate identified as an AmpC-producer were discharged. Nevertheless, in 24% of the patients the outcome was death (Figure 19).

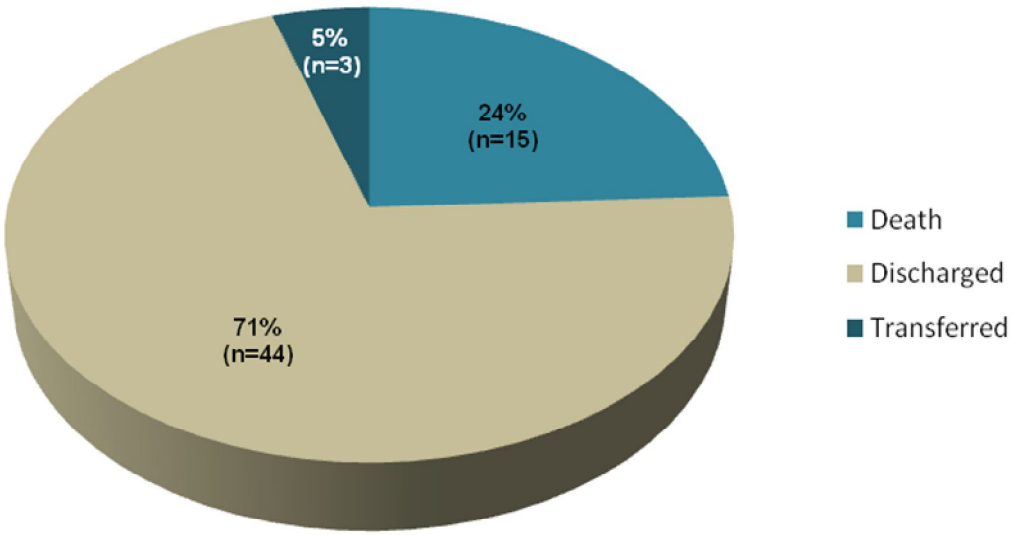

Figure 19 - Outcome of the patients carrying an isolate identified as an AmpC- producer.

Multidrug resistance is becoming a major problem observed in patients coming to the hospital, since it highlights the lack of control in dominating its spread throughout the community. From the AmpC positive diagnosed patients,

24% have died after visiting the hospital, presumably of the infection caused by the multidrug resistant bacteria.

Most patients carrying isolates identified as AmpC-producers suffer from co-morbidities (diabetes mellitus, chronic renal failure, abdomino-biliary diseases and neoplasia) and have undergone invasive procedures, such as insertion of urinary catheters (UC) or nasogastric tubes (NGT) or are mechanically ventilated, but both nosocomial- and community-related transmissions have been documented. Plasmid-mediated β-lactamases might spread by a horizontal gene transfer. (Conen, Frei *et al.* 2015)

4.3. General conclusion

Although AmpC-positive strains are less common than ESBL producers, its expression generally masks the presence of ESBLs, thus improving the probability of falsely reporting them as ESBL-negative. Consequently, the patient will not receive the appropriate medication and will possibly become the focus of new multiresistant strains, selected by wrong antibiotic administration.

Plus, this study identified a large number of strains carrying two types of resistance – both ESBL and AmpC – fact that enhances the need to use a combination of methods that reliably detects both mechanisms. Cefoxitin disc should be used along with the automated method, since it is consistently capable of differentiating these kinds of resistance mechanisms.

5. General Discussion

5.1 Epidemiology of ESBL producing isolates causing UTI in elderly (2013 – 2014)

In the present study, the percentage of isolates producing an ESBL is still high, being a motive of concern and it is necessary to adopt strict policies for the control of antibiotics administration in order to stop the spread of these isolates. As measure of control of ESBL-producers, some authors emphasize the need for culture of a mid-stream urine specimen prior to commencing antibacterials, especially in patients with the risk factors identified herein associated with ESBL-producers UTI/bacteriuria. (Osthoff, McGuinness et al. 2015)

The appearance of extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria causing community-acquired infections, including urinary tract infection, constitutes an important therapeutic challenge in choosing empirical antibiotics. (Cho, Jung et al. 2015; Osthoff, McGuinness et al. 2015) Antibiotherapy is also the reason for the emergence of resistance. Some studies have proven that patients with bacteremia caused by *Enterobacter spp* and treated with broad spectrum cephalosporins developed decreased susceptibility and augmented β-lactamase production after being treated with cefotaxime, ceftazidime or ceftizoxime. (Jacoby 2009)

Moreover, UTI caused by ESBL-producers is associated with significant clinical and economic burden. The cost of care and length of stay of patients carrying an ESBL-producer causing an UTI can be 1.5 to 1.7 times those caused by non-ESBL-producers. (Lee, Kotapati et al. 2006; MacVane, Tuttle et al. 2014) The cost of non-urinary tract infections caused by ESBL-EK was 1.7 times the cost of non-urinary tract infections caused by non-ESBL producers. Prompt recognition and appropriate antimicrobial selection may minimize this ESBL-related impact on hospital costs.

5.2 Epidemiology of AmpC producing isolates, in CHBV

AmpC β-lactamases are a major concern and clinically significant, since they confer resistance to cephalothin, cefazolin, cefoxitin, most penicilins and β-lactam/β-lactamase inhibitor combinations. Inhibitors such as clavulanic acid, sulbactam and tazobactam have low effect on AmpC β-lactamases. In fact, they can induce their expression. Cloxacillin, oxacillin and aztreonam are good inhibitors. (Jacoby 2009; Seral, Gude et al. 2012)

The laboratory detection of AmpCs can be complex and sometimes misleading. Also, pAmpC producers are difficult to identify by means of the routinely performed microbiological analyses and may be misclassified as ESBL producers and vice versa. (Robberts, Kohner et al. 2009; Munier, Johnson et al. 2010) Therefore, underreporting is likely. (Perez-Perez and Hanson 2002; Philippon, Arlet et al. 2002; Robberts, Kohner et al. 2009)

For pAmpC-related infections, data are lacking or include only small patient numbers. (Conen, Frei et al. 2015) Detection of AmpC β-lactamase in *Klebsiella spp*, *Salmonella spp*, *C. koseri* or *P. Mirabilis* is confirmatory of plasmid mediated AmpC production, because these organisms don't have a chromosomal AmpC β-lactamase. *E. coli* routinely carries cAmpC genes, which are very rarely hyper expressed, and therefore, *E. coli* commonly responds to second and third generation cephalosporins. (Conen, Frei et al. 2015) In *E. coli*, phenotypical detection of AmpC does not differentiate between chromosomal or plasmid-mediated enzymes: lack of multiple drug resistance is suggestive of a chromosomal AmpC, and multiple drug resistance is consistent either chromosomal or plasmid-mediated AmpC production. (Thomson 2010)

It is critical that ESBL, AmpC and carbapenemases are promptly identified, and this can only be achieved in a time consuming, extended confirmatory process. Microbiology labs are obviously being optimized by maximizing automatic systems, dangerously disregarding of personal interpretation. This approach

misleads the emerging complex antimicrobial profiles, which requires expert staff and additional methods.

6. General Conclusion

6. Conclusion

The results that are included in this thesis are of utmost importance, since they constitute a study carried out to analyze the epidemiology of β-lactamases in both the hospital environment and in the community. Therefore, this study provides a realistic panorama of the dissemination of β-lactamases in our region. Additionally, this study helps to highlight the importance of β-lactamases screening in order to prevent treatment failure.

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8. Appendices

8.1. Appendix 1

Abstract (online publication)

Epidemiology of ESBL-producing isolates causing UTI in the elderly (Aveiro, Portugal)

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Objectives: The world population is getting older and therefore more prone to acquire infections. Urinary tract infections (UTIs) are one of the most prevalent community-acquired infections. The aim of this study was to access the prevalence of ESBL-producing isolates collected from urine of elderly attending the Emergency Room (ER) during a 4 years period (2011-2014).

Methods: The bacterial strains were isolated from urine from patients, with primary diagnose UTI, older than 65 years, in ER during the period between 2011 and 2014. The Vitek2 system (according to CLSI 2012/EUCAST 2014guidelines) and Advanced Expert System (VITEK 2 AES) (BioMérieux, Marcy L´Étoile, France) were used to identify the isolates and to perform the antibiotic susceptibility profile of the isolates. ESBL production was confirmed using the Etest® (AB Biodisk) method, according to the manufacturer instructions.

Results: During the timeframe studied 1686 urine samples were collected following the criteria listed above. The percentage of *Escherichia coli* isolates has been increasing since 2011, comparing with the percentage of *Klebsiella pneumoniae* isolates, which in 2011 was higher than *E. coli* but has been significantly decreasing in the last three years (Table 1). Table 1, also shows a reduction in the percentage of ESBL-producers until 2013, however in 2014 the percentage increased. In 2011, 82,5% of *K. pneumoniae* isolates were ESBL-

producers, and progressively decreases until 2013. However in 2014, the percentage slightly increases to 44,1%. *E. coli* ESBL-producers have also progressively diminished until 2013, exhibiting an increase (18,0%) in 2014.

Conclusions: In the last three years *E. coli* was the organism causing higher number of UTI. However, the percentage of *K. pneumonia* ESBL-producers was always higher than *E. coli.* Considering the criteria used to select the isolates we can conclude that they are disseminated in community, mainly in nursing homes, but also in patients who attend the ER repeatedly. Despite the percentage of ESBL producers has been significantly reduced in the last three years, the values are still high, being a motive of concern specially considering the elderly population.

Table. 1 Number of isolates selected, percentage of *K. pneumoniae* and *E.coli* identified, and percentage of ESBL producers respectively.

	Clinical Isolates Selected	% *K. pneumoniae*	% *E. coli*	% *K. pneumoniae* ESBL	% *E. coli* ESBL
2011	272	35.7	29.8	82.5	44.4
2012	384	19.0	48.4	60.4	15.6
2013	506	18.4	45.7	38.7	12.1
2014	524	15.1	46.8	44.1	18.0

8.2. Appendix 2

Abstract (online publication)

Surveillance of MDR Gram-negatives ESBL-producers and carbapenem resistant, in 12 years period (2003-2014) in Aveiro, Portugal

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Objectives: Multidrug resistance has become a burden to the health care system. The present study was undertaken to determine the prevalence of multidrug resistant Gram-negatives (fermenters ESBL-producers and non-fermenters carbapenem resistant) in a 12 years timeframe, in Centro Hospitalar do Baixo Vouga (CHBV), Aveiro, Portugal.

Methods: During the timeframe studied, consecutive, non-duplicate bacterial pathogens were isolated from various clinical specimens, from inpatients and outpatients and exhibiting resistance to at least three different classes of antibiotics. Four prevalent species were selected, namely *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP)*, Pseudomonas aeruginosa* (PA) *and Acinetobacter baumannii* (AB). Identification of the isolates was performed with the Vitek2 system and Advanced Expert System (VITEK 2 AES) (BioMérieux, Marcy L´Étoile, France). Antimicrobial susceptibility profile to >20 antimicrobial agents was evaluated according to CLSI 2012/EUCAST 2014 guidelines. ESBL producers were confirmed by Etest (AB Biodisk) ESBL with cefotaxime/cefotaxime + clavulanic acid and ceftazidime/ceftazidime + clavulanic acid strips, according to manufacturer's instructions.

Results: 14209 isolates were included in this study, according to the criteria listed above. 11109 were fermenters, 8639 belong to EC species and 2425 belong

to KP species. Among these, a total of 11% (1549) isolates were ESBL producers. An ESBL phenotype was detected in 36% (869/2425) of the KP and 8% (680/8639) of the EC. Comparing both species, the number of KP ESBL-producers was most of the time higher than EC ESBL-producers. 3100 were non-fermenters, 2446 belong to PA species and 654 belong to AB species. It was observed an increase of resistance rates to carbapenems: meropenem and imipenem. Resistance to colistin on the other hand did not show significative variation.

Conclusion: KP isolates are a major concern since despite the total number being inferior to *E. coli*, the number of ESBL-producers belonging to that species is higher. The number of ESBL-producing isolates of these species has been significantly increasing in the last 12 years and requires surveillance. The increase of carbapenem resistance exhibited, in the last 5 years by the non-fermenters, was extremely high and compromises the use of these antibiotics to treat infections caused by these pathogens. This fact can be explained by the presence of an integron carrying a VIM gene, among the PA isolates in our hospital. Also, long-term dissemination of a blaOXA-40 producer *A. baumannii* in the Iberian Peninsula has been reported by da Silva and co-workers, thus unsurprisingly it was detected in our hospital. However, this limits the therapeutic options available, thus becoming a cause for concern. Colistin remains the most active drug against both PA and AB in the isolates collected in CHBV.